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COUPLING PROCESS, IN SOLUTION, BETWEEN A PEPTIDE AND AT LEAST ONE OTHER COMPOUND, AND ITS APPLICATIONS.

The present invention relates to a coupling process, in solution, between a peptide and at least one compound bearing a carboxylic acid or alcohol function, such as a lipid, a sugar, an alcohol or a fluorescence marker, as well as to modified peptides which are essentially constituted by a peptide linked, by a hydrazide bond, to at least one compound as defined above.

The present invention also relates to the use of N,N'-tri(Boc)hydrazinoacetic acid or N,N'-di(Boc)hydrazinoacetic acid for functionalizing 10 a peptide with a α -hydrazinoacetic group.

The problem of the entry of living cells by different substances possessing pharmacological properties is of considerable importance therapeutically. Synthetic peptides and oligonucleotides have difficulty in passing through the cellular membrane. One interesting approach aimed at improving their ability to penetrate a 15 cell is that of modifying them with a lipophilic part. It has thus been shown that a peptide modified by a simple aliphatic chain is capable of penetrating the cell by passive transfer through the membrane, and of interacting with its intracytoplasmic target. Lipopeptides are thus molecules of interest for the purpose of vectorizing a functional pattern within the cell.

20 Lipopeptides can be synthesized, for example, by coupling a fatty acid with a peptide in solid phase, as described, in particular, by K. THIAM *et al.* in *Biochemical and Biophysical Research Communications*, 1998, 253, 639-647. Upon completion of synthesis, steps of cleavage the peptide / solid support bond and deprotection of the side chains of the peptide by a strong acid have to be carried out. 25 This treatment considerably restricts the choice of the lipophilic part; it prohibits, in particular, the use of unsaturated fatty acids. Furthermore, purification of the lipopeptides by reverse phase high-performance liquid chromatography is difficult and leads to low yields, given the numerous impurities that are present at the end of synthesis.

30 It has also been proposed coupling, in solution, a protein with a palmitoyl-coenzyme A group, the latter being introduced in the thiol group of a cysteine. Such a coupling leads to the formation of a thioester link, which has the drawback of being unstable. Furthermore, this strategy is confined to the modification of certain proteins by palmitoyl-coenzyme A and cannot be generalized for the 35 synthesis of lipopeptides.

Present strategies for the synthesis of lipopeptides also involve using

chemical ligation reactions. Chemical ligation makes it possible to link, in solution and under extremely mild conditions, two peptide structures that have been previously purified and completely deprotected.

It has thus been proposed to link a fatty acid to a peptide with a 5 disulphide link, in an aqueous buffer solution. However, the disulphide link possesses numerous problems; such a link is, in fact, unstable and liable to be degraded in the presence of thiols, whence the need to avoid contaminating the solvents used to solubilize the products with thiols, as well as the impossibility of introducing a 10 cysteine into the peptide sequence to be vectorized. The use of the thiol chemistry further necessitates working in an inert atmosphere in order to prevent oxidization of the thiols.

W. ZENG *et al.* (*J. Pept. Sc.*, 1996, 2, 66-72) have also proposed coupling, in solution, a peptide that has been completely deprotected and previously purified to a polyfunctional lipidic structure linked to a peptide, this being effected via 15 an oxime link. The lipophilic part is introduced into a peptidic sequence in solid phase, such a method having the aforementioned drawbacks, namely limitation as to the choice of the lipophilic part and the difficulties associated with purification of the lipidic structure.

Similarly, O. MELNYK *et al.* (*J. Peptide Res.*, 1998, 52, 180-184) 20 have described ligation, in solution and by a hydrazone link, between a peptide bearing a lipophilic chain and an aldehyde function and another peptide modified at the lysine side chain by a hydrazino group. The hydrazone link is produced in solution, but the lipophilic compound, which is of a peptidic nature, is solid phase synthesized, and the limitations are the same as those described earlier.

Furthermore, C. KLINGUER *et al.* (*Tetrahedron Letters*, 1996, 37, n° 40, 7259-7262) describe ligation, in a water / acetonitrile mixture and by a 25 hydrazone link, between a peptide bearing a hydrazine function and cyclohexane-carboxaldehyde. The process described by these authors does not, however, make it possible to obtain compounds that are stable, hence usable for vectorizing active 30 principles: indeed, the hydrazone link formed between the hydrazine and the aldehyde is unstable over a large range of pH values.

Chemical ligation appears to be an excellent method for the 35 synthesis of lipopeptides permitting an improvement in yields obtained for these compounds. However, we have seen that there are no ligation methods, at the present time, not using thiol chemistry and permitting direct coupling of a lipophilic compound, non linked to a carrier structure, to a completely deprotected peptide.

The Inventors thus assigned themselves the task of providing a new strategy for the synthesis of lipopeptides and, in general, of peptides modified by different compounds of a lipidic, or other, nature, by chemical ligation in solution.

5 This new synthesis strategy must, in particular, meet the following criteria:

- the coupling of the above-mentioned compound, for example a lipid, to the peptide, takes place in solution,
- coupling is carried out using a peptide that has been completely deprotected, the reaction being chemoselective,
- 10 - the reaction conditions of coupling permit the direct use of fatty acids and of commercial cholesterol derivatives,
- the reaction conditions of coupling permit, in particular, the introduction, onto the peptide, of carboxylic acids and sensitive alcohols such as, for example, mono- and polyunsaturated complex fatty acids and cholesterol derivatives,
- 15 - the link formed during coupling is very stable over a large range of pH values.

The Inventors also assigned themselves the task of providing modified peptides, capable of being obtained by chemical coupling, wherein said peptides are linked to different compounds, in particular lipids, by a very stable link 20 not having the drawbacks of the disulphide links of the prior art.

These objectives are obtained by creating a hydrazide link between the peptide and the compound that is linked thereto, during convergent synthesis in solution.

25 The present invention relates to a coupling process between a peptide and at least one compound A, of a non-peptidic nature, bearing a function selected from the group formed by the carboxylic acid functions and the alcohol functions, characterized in that said coupling includes a step of producing, in solution, a hydrazide link between said peptide and said compound A.

Within the meaning of the present invention, the word "peptide" is 30 to be taken as meaning any coupling of several amino acids, whatever their nature and number; the term "peptide" thus refers both to oligopeptides (dipeptides ou tripeptides) and polypeptides or proteins.

In a particularly advantageous way, the process according to the invention, which is carried out in solution, makes it possible to avoid a step of 35 cleavage of the modified peptide obtained from the support, which cleavage, as we have already seen, considerably restricts the choice of the compound linked to said

peptide. Furthermore, the hydrazide link produced between the peptide and the compound or compounds A is very stable, this being the case over a very wide range of pH values, and *in vivo*.

According to one advantageous form of embodiment of the coupling process according to the present invention, the latter includes, for the purpose of producing said hydrazide link, the following steps :

- 5 a) activation of the function borne by said compound A into a corresponding reactive function, selected respectively from the group formed by the ester functions and the carbonate functions, when compound A bears, respectively, a carboxylic acid function and an alcohol function; and
- 10 b) reaction, in solution and at a pH of less than 6, between said compound A activated obtained in a) and a peptide, that is completely deprotected, bearing at least one hydrazine or hydrazine derivative group, either at its N-terminal end or at the end of the side chain of a lysine or of an ornithin possibly present at 15 some point in the peptide sequence.

Within the meaning of the present invention, a "hydrazine group" is to be taken as meaning a formula -NH-NH₂ group. A "hydrazine derivative group" is to be taken as meaning any group comprising at least the structure -NR-NH₂ (including, advantageously, the structure -CO-CHR₁-NR-NH₂), where R₁ and R are, 20 independently of one another, a hydrogen atom or a saturated or unsaturated, linear, branched or cyclic alkyl group, including from 1 to 10 carbon atoms as well as, possibly, 1 to 3 heteroatoms chosen from oxygen, sulfur and nitrogen, and substitutable by 1 to 6 groups chosen from the hydroxy, alkoxy, aryloxy, amino, aminoalkyl, aminoaryl, thio, thioalkyl, carbonyl, carboxyl, guanidino et carboxamido 25 groups.

The hydrazide link formed in the course of the coupling process according to the invention thus includes at least the structure -CO-NH-NR-, R being as defined above.

A hydrazine group can be introduced either at the N-terminal end of 30 the peptide or at the end of the side chain of a lysine or an ornithin possibly present at any point in the peptide sequence, by any means known to a person skilled in the art, for example according to an N-amination protocol as described by C. KLINGUER *et al.* in Tetrahedron Letters, 1996, 37, 40, 7259-7262.

In a particularly advantageous way, the coupling reaction between 35 said activated compound A and said completely deprotected peptide, functionalized as described above, makes it possible to avoid any step of deprotecting the side chains of

the peptide with a strong acid following the coupling reaction, which makes it possible to use, as compound A, sensitive fatty acids. The process according to the invention thus makes it possible to obtain the modified peptide, that is to say the peptide linked to compound A, directly.

5 The process according to the invention makes it possible to effect a chemoselective reaction between the functional group (the hydrazine group or hydrazine derivative group) introduced into the peptide and the activated compound or compounds A; the reaction takes place, in fact, at a pH lower than 6, a pH such that the amino functions of the side chains of the lysines (ϵ -NH₂ function) or the ornithines (δ -NH₂ function) or the N-terminal α -NH₂ function possibly present in peptide sequence are protonated, hence non-reactive. Control of the pH thus makes it possible to preferentially acetylate the hydrazine or hydrazine derivative group introduced into the peptide, without the other functional groups of the side chains of the amino acids constituting the peptide reacting.

10 15 The coupling reaction carried out during the process according to the present invention (step b) takes place under very mild operating conditions and, in a particularly advantageous way, does not necessitate working under inert conditions, as is the case with certain processes of the prior art, in particular those that consist in coupling a peptide to a fatty acid with a disulphide link.

20 According to one advantageous form of embodiment of the coupling process according to the invention, said process further includes a step c) of purification of the modified peptide obtained in step b).

25 Such purification is, conventionally, carried out using high-performance liquid chromatography. By comparison with the purification of a modified peptide obtained with a coupling process carried out in solid phase, as described above, purification of the modified peptide obtained with the coupling process according to the present invention leads to far better yields, with the modified peptide obtained in step b) being purer than a modified peptide obtained in solid phase.

30 According to another advantageous form of embodiment of the coupling process according to the present invention, after step a) of activation of the function borne by compound A, the corresponding reactive function borne by compound A is selected from the group constituted by succinimidyl, sulfosuccinimidyl and aryl esters and carbonates.

35 Para-nitrophenyl esters and carbonates can be cited as examples of aryl esters and carbonates.

According to another advantageous form of embodiment of the coupling process according to the invention, said hydrazine derivative group borne by the peptide is an α -hydrazinoacetic group (a group having the formula -CO-CH₂-NH-NH₂).

5 According to one preferred arrangement of this form of embodiment, prior to step b) of the process according to the invention, said peptide is functionalized by an α -hydrazinoacetic group, either at its N-terminal end or at the end of the side chain of a lysine or of an ornithine possibly present at some point in the the peptide sequence, using N,N'-tri(Boc)hydrazinoacetic acid or 10 N,N'-di(Boc)hydrazinoacetic acid.

According to one preferred modality of this arrangement, the functionalization of said peptide by an α -hydrazinoacetic group, by means of N,N'-tri(Boc)hydrazinoacetic acid or N,N'-di(Boc)hydrazinoacetic acid, is followed by a step of purification of said peptide functionalized using high-performance liquid 15 chromatography, using an eluent constituted by a water / alcohol mixture, preferably a water / isopropanol mixture, including trifluoroacetic acid. Such an eluent advantageously makes it possible to avoid any degradation of the α -hydrazinoacetic group borne by the peptide.

According to another advantageous form of embodiment of the 20 coupling process according to the invention, said compound A is selected from the group formed by lipids, sugars, alcohols and fluorescence markers.

As an example of a usable fluorescence marker, mention can be made, non-limitatively, of fluorescein or rhodamine.

According to one preferred arrangement of this form of 25 embodiment, said lipids are selected from the group formed by saturated fatty acids, unsaturated fatty acids and sterols. The process according to the invention advantageously makes it possible, in fact, to link to a peptide complex (mono- and polyunsaturated) fatty acids and, generally speaking, any sensitive carboxylic acid. Preferably the above-mentioned lipids are selected from the group constituted by 30 palmitic acid, stearic acid, cis-9,10-epoxystearic acid, oleic acid, linoleic acid and cholesterol.

The present invention also relates to a modified peptide essentially constituted by a peptide linked, by a hydrazide link, to at least one compound A bearing, before its link to said peptide, a function selected from the group formed by 35 carboxylic acid functions and alcohol functions.

According to an advantageous embodiment, the modified peptide

according to the present invention is essentially constituted by a peptide linked, by a hydrazide link, to at least one compound selected from the group formed lipids, sugars, alcohols and fluorescence markers.

According to one preferred arrangement of this form of
5 embodiment, the modified peptide according to the present invention is an oligopeptide essentially constituted by a peptide linked, by a hydrazide link, to at least one lipid selected from the group constituted by saturated fatty acids, unsaturated fatty acids and sterols.

Preferably, said oligopeptide according to the invention is essentially
10 constituted by a peptide linked, by a hydrazide link, to at least one lipid selected from the group constituted by palmitic acid, stearic acid, cis-9,10-epoxystearic acid, oleic acid, linoleic acid and cholesterol.

The stability of a hydrazide link makes the modified peptides
according to the invention particularly valuable since the hydrazide link is stable both
15 *in vivo* and over a very wide range of pH values. Furthermore, the hydrazide link is stable under catalytic hydrogenation conditions, which permits, for example, in the case of peptides modified by unsaturated fatty acids, the synthesis of lipopeptides marked with tritium in the fatty chain, useful for an intracellular radioactive follow-up of said lipopeptides and better understanding of their mechanism of action.

20 The present invention also relates to a synthetic vaccine and a diagnosis reagent which include at least a modified peptide according to the present invention, as described above.

The present invention also relates to the use of the coupling process
according to the invention, as described above, for the preparation of a medicament
25 including an active principle of a vectorized peptidic type, useful for cellular targeting.

The present invention further relates to the use of N,N'-tri(Boc)hydrazinoacetic acid or N,N'-di(Boc)hydrazinoacetic acid for functionalizing
a with an α -hydrazinoacetic acid, either at the N-terminal end of said peptide or at the
30 end of the side chain of a lysine or of an ornithin possibly present at some point in the peptide sequence.

It is clearly understood, however, that an α -hydrazinoacetic group
can be introduced into said peptide either at the N-terminal end of said peptide or at
the end of the side chain of a lysine or of an ornithin possibly present at any point in
the peptide sequence using any process known to a person skilled in the art; for
35 example, functionalization of a peptide with an α -hydrazinoacetic group can be
carried out via a solid phase N-amination reaction, as described by C. KLINGER

et al. in *Tetrahedron Letters*, 1996, 37, 40, 7259-7262, by means of the commercial reagent N-Boc-3-(4-cyanophenyl)oxaziridine (BCPO). This is the case, for example, of an N-amination reaction carried out on a glycine residue in the N-terminal position of a peptide or on the side chain of a lysine or of an ornithine present at some point in 5 the peptide sequence.

However, given the high cost of the BCPO and the very considerable time taken by such a reaction, this method of synthesis is suitable only for the functionalization of products with high added values, synthesized in small quantities. In a particularly advantageous way, the use of N,N'-tri(Boc)-10 hydrazinoacetic acid or of N,N'-di(Boc)hydrazinoacetic acid according to the present invention is simpler and far less expensive for functionalizing a peptide with an α -hydrazinoacetic group. This functionalization is carried out in solid phase, the functionalized peptide then being separated from the solid support and deprotected using methods known to a person skilled in the art; a step of purification using high-15 performance liquid chromatography can then be carried out, using the water / alcohol eluent already described, which advantageously enables any degradation of the α -hydrazinoacetic group borne by the peptide to be avoided.

Apart from the above arrangements, the invention also includes other arrangements which will emerge from the following description, which refers to 20 examples of embodiments of the process of the present invention and of syntheses of modified peptides according to the present invention, as well as to the annexed drawings, in which:

- Fig. 1 illustrates the synthesis of N,N'-tri(Boc)hydrazino-acetic 4 and N,N'-di(Boc)hydrazinoacetic 4' acids;
- 25 - Fig. 2 illustrates the synthesis of a hydrazinopeptide 6 from a peptide 5 and N,N'-tri(Boc)hydrazinoacetic acid;
- Fig. 3 illustrates the synthesis of lipopeptides 11, 12, 13, 14, 16 and 18 according to the process according to the present invention, from hydrazinopeptide 6 and lipids 7, 8, 9, 10, 15 and 17, with Su representing a succinimidyl group;
- 30 - Fig. 4 illustrates the synthesis of lipopeptide 13 by catalytic hydrogenation of lipopeptide 12;
- Fig. 5 illustrates the synthesis of lipopeptide 21 using the process according to the present invention;
- Fig. 6 illustrates the synthesis of lipopeptide 23 using the process 35 according to the present invention.

It should be clearly understood, however, that these examples are

given solely by way of illustration of the object of the invention, of which they in no way constitute a limitation.

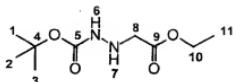
In the examples that follow, the following abbreviations are used:
 eq.: equivalents; Boc: *tert*-butyloxycarbonyl; Boc₂O: di(*tert*-butyloxycarbonyl) ether;
 5 CH₂Cl₂: dichloromethane; AcOH : acetic acid; AcOEt: ethyl acetate; Na₂SO₄: sodium sulfate; KH₂PO₄: potassium dihydrogenophosphate; Na₂HPO₄: disodium phosphate; DMF: dimethylformamide; DMAP: 4-dimethyl-aminopyridine; PEG: polyethyleneglycol; PS: polystyrene; CDCl₃: deuterated chloroform; CD₃CO₂H : acetic acid d₃; TFA: trifluoroacetic acid; Et₂O: diethylether; EDT: ethanedithiol;
 10 NMP: N-methylpyrrolidone; THF: tetrahydrofuran; HBTU: N-oxide of N-[(1H-benzotriazol-1-yl) (dimethylamino) methylene]-N-methylmethanaminium hexafluorophosphate; HOBT: N-hydroxy-benzotriazole; *t*Bu: *tert*-butyl; DIEA: diisopropylethylamine; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulphonyl; Trt: trityl; Fmoc: 9-fluorenylmethoxy-carbonyl; Pbf: 2,2,4,6,7-15 pentamethyldihydro-benzofuran-5-sulfonyl; BOP: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate; HPLC: high-performance liquid chromatography; RP-HPLC: reverse phase high-performance liquide chromatography; ES-MS: electrospray mass spectrometry; TOF: time-of-flight; MALDI: matrix-assisted laser desorption ionisation; NMR: nuclear magnetic 20 resonance; TOCSY: total correlation spectroscopy ; PDMS: Plasma desorption mass spectrometry; PAL: peptide-amide linker.

EXAMPLE 1: Synthesis of N,N'-tri(Boc)hydrazinoacetic 4 and N,N'-di(Boc)hydrazinoacetic 4' acids (Fig. 1).

1) Synthesis of N,N'-tri(Boc)hydrazinoacetic 4 acid

25 • Synthesis of N-Boc ethyl hydrazinoacetate 2
 14.9 g (96.4 mmoles) of commercial ethyl hydrazinoacetate hydrochloride 1 and 26.1 g (119.5 mmoles) of Boc₂O are dissolved in 70 ml of a water / ethanol mixture (1/1). After dissolution of the reagents, the reaction medium is cooled down to 0°C. 13.2 ml of N-methylmorpholine (119.5 mmoles) are then added 30 to the reaction mixture. After stirring for 15 minutes at 0°C, followed by 2h at ambient temperature, the mixture is diluted in 100 ml of water. The aqueous phase is saturated with KH₂PO₄, and then extracted with diethylether (3 x 70 ml) and petroleum ether (2 x 70 ml). The organic phases are collected and then dried on anhydrous Na₂SO₄, and finally concentrated at reduced pressure. The yellow oil obtained (19.8 g, 91.1 35 mmoles, yield: 94.5%) is dried on phosphorus pentaoxide (P₂O₅) overnight. Product 2 thus obtained, represented below, is used without any other form of purification in the

remainder of the synthesis.



5 The analysis of product 2 is as follows: RMN ^1H (CDCl_3 , ref TMS, 300 MHz, 323 K) δ : 4.19 (q, 2H, H_{10-11} =7.18 Hz), 4.11 (s, 2H, H_8), 1.45 (m, 9H, $\text{H}_{1,2,3}$), 1.26 (t, 3H, H_{11} , J_{11-10} =7.16 Hz). RMN ^{13}C (CDCl_3 , TMS, 75 MHz, 323 K) δ : 175.51 and 174.38 (C_3), 161.76 and 160.83 (C_9), 85.89 and 85.23 (C_4), 65.57 and 65.46 (C_{10}), 57.41 (C_8), 32.87, 32.78 and 32.63 (C_1 , C_2 , C_3), 18.70 (C_{11}). Elementary analysis calculated for $\text{C}_9\text{H}_{18}\text{N}_2\text{O}_4$: C 49.53; H 8.31; N 12.84; O 29.32; found: C 49.78; H 8.36; N 12.33; O 29.27.

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• *Synthesis of ethyl N,N'-tri(Boc)hydrazinoacetate 3*

Compound 2 (19.86 g, 91.1 mmoles) is dissolved in 16 ml of CH_2Cl_2 in an inert atmosphere and in the presence of 38.5 ml of Et_3N (276 mmoles) at 15 0°C. Furthermore, 60.2 g (276 mmoles) of Boc_2O are dissolved in 20 ml of CH_2Cl_2 in the presence of 3.4 g (27.6 mmoles) of DMAP at 0°C. After complete dissolution of the reagents, the composite 2 / Et_3N mixture is added dropwise to the Boc_2O / DMAP mixture in an inert atmosphere and at 0°C. Once the addition has been completed, the temperature of the reaction mixture is gradually restored to ambient temperature. After 20 stirring for 2h, the medium is diluted with 50 ml of CH_2Cl_2 . The organic phase is washed with a solution saturated with KH_2PO_4 (3 x 75 ml), dried on sodium sulphate, and then concentrated at reduced pressure. The residual yellow-orange oil is purified by filtering on silica (40-60 microns, 160 g) with a CH_2Cl_2 / AcOEt mixture (97:3). The residual yellow oil is dried overnight in the presence of P_2O_5 . 36.9 g 25 (88.3 mmoles; yield: 96.9%) of product 3 are thus obtained.

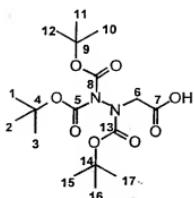
The analysis of product 3 using NMR is as follows: NMR ^1H (CDCl_3 , ref TMS, 300 K) δ : 4.16 (s, 2H), 3.71 (q, 2H, J = 7 Hz), 1.46 (m, 27H), 1.23 (t, 3H, J =7 Hz). NMR ^{13}C (CDCl_3 , ref TMS, 300 K) δ : 167.74 ($\text{C}=\text{O}$), 150.48 and 150.23 ($\text{C}=\text{O}$), 83.68, 82.46 and 82.00 (quaternary C), 60.98 and 58.40 (OCH_2CH_3), 30 53.51 and 51.57 (CH_2CO), 28.09 (CH_3C), 18.42 and 14.20 (CH_2CH_3).

Elementary analysis of product 3 (empirical formula: $\text{C}_{19}\text{H}_{34}\text{N}_2\text{O}_8$): C 54.53, H 8.19, N 6.69 (calculated), C 54.81, H 8.25, N 6.71 (found).

Analysis of product 3 by mass spectrometry: MALDI-TOF $[\text{M}+\text{H}]^+$ calculated: 419.5, found: 441.4 $[\text{M}+\text{Na}]^+$, 457.4 $[\text{M}+\text{K}]^+$.

• *Synthesis of N,N'-tri(Boc)hydrazinoacetic acid 4*

Compound 3 (15.05 g, 36.01 mmoles) is dissolved in 40 ml of 100% pure ethanol. The medium is cooled down to 0°C and 39.6 ml (39.6 mmoles) of a molar soda solution at 0°C are added dropwise. After 25 minutes' stirring at 0°C, the reaction medium is neutralized by the addition, dropwise, of 20 ml of a citric acid solution (634 mg/ml) to a pH of 4.0. The mixture is then diluted with 50 ml of water and extracted with diethyl ether (2 x 80 ml) and then dichloromethane (2 x 80 ml). The organic phases are collected, washed with a solution saturated with NaCl (2 x 40 ml), and then dried on anhydrous Na₂SO₄, filtered, and finally concentrated at reduced pressure. The residual oil is cold precipitated in a diethyl ether (55 ml)/heptane (74 ml) mixture. Product 4 obtained, represented below, is a white solid (9.63 g, yield: 68.5%).



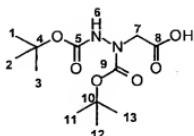
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The analysis of product **4** is as follows: NMR ^1H (DMSO-*d*₆, ref TMS, 300 MHz, 300 K) δ : 4.04 (s, 2H, H₆), 1.42 (m, 27H, H_{1-3,10-12,15-17}). NMR ^{13}C (DMSO-*d*₆, ref TMS, 75 MHz, 300 K) δ : 169.3 (C₇), 153.5 and 49.8 (C_{5,13,8}), 82.9 and 81.7 and 81.1 (C_{4,9,13}), 53.1 and 51.2 (C₆), 27.8 (C_{1-3,10-12,15-17}). Elementary analysis calculated for C₁₇H₃₀N₂O₈: C 52.30; H 7.74; N 7.17; O 32.78; found: C 52.26; H 7.77; N 7.22; O 32.63.

2) Synthesis of N,N'-di(Boc)hydrazinoacetic acid 4'

Compound 3 (36.9 g, 88.3 mmoles) dissolved in 135 ml of ethanol is treated with 135 ml of molar soda at 0°C. After 30 minutes' stirring at 0°C, the temperature of the reaction mixture is gradually restored to ambient temperature. The mixture is then stirred for 3h30 at ambient temperature. The reaction medium is then diluted with 110 ml of water and extracted with diethyl ether (2 x 80 ml). The aqueous phase is acidified with the addition of hydrochloric acid 1 N to a pH of 2. The reaction is exothermal. The aqueous phase is then extracted with dichloromethane (2 x 80 ml) and then diethyl ether (2 x 80 ml). The organic phases are collected, washed with a

solution saturated with KH_2PO_4 , dried on anhydrous Na_2SO_4 , filtered and, finally, concentrated at reduced pressure. The residual mixture is kept overnight at 4°C and then it is cold-recrystallized in a diethyl ether/heptane mixture (90 ml/120 ml). Product 4' obtained, represented below, is a white solid (17.9 g, 61.6 mmoles, yield: 5 69.8%).



The analysis of product 4' is as follows: NMRH (DMSO-*d*₆, ref 10 TMS, 300 MHz, 300 K) δ : 9.23 (s, 0.53H, H₅), 9.16 (s, 0.26H, H₆), 8.84 (s, 0.14H, H₆), 8.74 (s, 0.07H, H₆), 3.97 (s, 2H, H₇), 1.39 (m, 18H, H_{1-3,11-13}). RMN ¹³C (DMSO-*d*₆, ref TMS, 75 MHz, 300 K) δ : 170.10 (C₈), 155.04 and 154.19 (C_{5,9}), 80.15 and 79.54 (C_{4,10}), 54.4 and 53.1 (C₇), 27.93 (C₁₋₃, C₁₁₋₁₃). Elementary analysis calculated for C₁₂H₂₂N₂O₆: C 49.65; H 7.64; N 9.65; O 33.07; found: C 50.09; H 7.84; N 9.57; 15 O 32.64.

EXAMPLE 2 : Synthesis and purification of hydrazinopeptide 6 (Fig. 2).

• Synthesis of hydrazinopeptide 6

Peptide 5 is produced on a Wang resin (0.73 mmol/g, Applied Biosystems, Foster City, USA), using the Fmoc/*tert*-butyl strategy, as described, for 20 example, by FIELDS *et al.* in *Int. J. Pept. Protein*, 1990, 35, 161, and HBTU/HOBt activation (see SCHNÖLZER *et al* in *Int. J. Pept. Protein Res.*, 1992, 40, 180), using a 431A Applied Biosystem peptide synthesizer (Foster City, USA). Protection of the side chains is provided by: His(Trt), Glu(O'Bu), Arg(Pmc), Lys(Boc). Upon completion of synthesis, the Fmoc group of the α -NH₂ function of the arginine is 25 displaced in the presence of 20% piperidine in the DMF. The N,N'-tri(Boc)hydrazinoacetic acid 4 (1.2 eq) is then introduced manually using BOP activation *in situ* (BOP 1.2 eq, DIEA 3.6 eq in the DMF for 20 minutes), as described, for example, by GAIKI *et al.* in *Tetrahedron Letters*, 1990, 50, 7363. Alternatively, 30 N,N'-di(Boc)-hydrazinoacetic acid could also be used. The peptidyl-resin is washed successively with DMF, dichloromethane, and then with ether. It is then dried at reduced pressure for 30 minutes.

Cleavage of the peptide link, as well as deprotection of the side chains, is carried out in the presence of a TFA/H₂O/anisole mixture (1 g of dry resin / 9.5 ml of TFA/0.25 ml of anisole/0.25 ml of H₂O) with stirring for 2h at ambient temperature. Peptide 6 is precipitated in an Et₂O/heptane mixture (1/1) previously 5 cooled down to 0° C (200 ml). The precipitate is centrifuged and then dissolved in an H₂O/AcOH mixture (5/1), deep frozen and freeze dried.

- Purification of hydrazinopeptide 6

The hydrazinopeptide 6 was purified using HPLC in a hyperprep C18 column using a linear gradient of 0% to 50% of a TFA/water /isopropanol 10 mixture (water / isopropanol ratio 2/3, with the mixture including 0.05% of TFA) in an 0.05% TFA/water mixture. Such an eluent advantageously makes it possible to prevent any degradation of the peptide. The purified compound is freeze dried and stored at -20°C.

The purity of the purified compound is checked using analytical 15 HPLC in a Vydac C18 column using the same eluent system as before. The identity of peptide 6 was checked by ES-MS analysis using a Micromass Quattro spectrometer ([M+H]⁺ calculated 1432.5, found 1432.7).

EXAMPLE 3 : Synthesis of lipopeptides 11, 12, 13, 14, 16 and 18 (Fig. 3).

20 1) Synthesis of compounds 8, 9, 10, 15 and 17
• Synthesis of compounds 8, 9, 15 and 17.

In the event of R (Fig. 3) representing the fatty chain of an oleic acid, 10 mg (35.4 µmoles) of oleic acid, 4.08 mg (35.4 µmoles) of N-hydroxysuccinimide and 4.3 µl (27.2 µmoles) of diisopropylcarbodiimide are dissolved in a THF/dichloromethane mixture (175 µl / 175 µl). After being left 25 overnight at 0°C, the medium is concentrated at reduced pressure. The residual oil (compound 8) is resuspended in 6.8 ml of 2-methyl-propene-2-ol.

The same procedure is used to activate the stearic, linoleic and cis-9,10-epoxystearic acids, that is to say to obtain these acids in the form of succinimidyl esters (obtaining compounds 9, 17 and 15).

30 • Synthesis of compound 10.

500 mg (1.13 mmoles) of cholesteryl chloroformate and 140.9 mg (1.22 mmoles) of N-hydroxysuccinimide are dissolved in 2 ml of dichloromethane at ambient temperature. 170 µL (1.22 mmoles) of triethylamine are added to the reaction medium. The reaction is exothermal and a white precipitate forms. After stirring for 35 45 minutes at ambient temperature, the medium is diluted with 50 ml of dichloromethane and washed with a saturated solution of KH₂PO₄. The organic phase

is dried on sodium sulphate, filtered and then concentrated at reduced pressure. Compound 10 obtained is a white solid (451.6 mg, 0.85 mmoles, yield: 76%). This is a cholesteryl carbonate activated with N-hydroxysuccinimide.

2) Synthesis of lipopeptide 11

5 6 mg (3 μ moles) of hydrazinopeptide 6, the synthesis of which is described in example 2, are dissolved in 900 μ l of a phosphate/citrate buffer, 0.25 mM, pH=5.2 (160.2 μ l of an 0.2 M Na₂HPO₄ solution and 139.8 μ l of citric acid, 0.1 M, topped up to 1.2 ml with water). The pH of hydrazinopeptide 6 in solution is re-adjusted if necessary with the Na₂HPO₄ solution, 0.2 M. 1.48 mg (3.6 μ moles) of 10 succinimidyl palmitate 7 (Sigma) are dissolved in 900 μ l of 2-methyl-propane-2-ol. The two solutions are then mixed and stirred at ambient temperature for 72 h.

15 The use of a mixed buffer /2-methyl-propane-2-ol medium makes it possible both to control the pH of the reaction medium and to ensure the proper solubility of the hydrazinopeptide 6, fatty acid 7 and final lipopeptide 11. In addition, the introduction of the lipophilic part into the peptide takes place under mild conditions, thus permitting the introduction of sensitive fatty acids into the strong acids.

20 The progress of the reaction is monitored by HPLC in a Zorbax C3 column (0 to 100% of solvent B at 0.05% TFA/80% acetonitrile/20% water in 30 minutes and then 5 minutes at 100% of solvent B, 1 ml/minute, detection at 215 nm). After 72 h, monitoring by HPLC shows that the reaction has ended. The reaction medium is then diluted with 5 ml of a water / acetic acid mixture (80/20) and purified in a Zorbax C3 column using the previous eluent system. After freezing and freeze drying, lipopeptide 11 is obtained with a yield of 61% (3.89 mg, 1.83 μ moles). Only 25 6% of diacylated lipopeptides are obtained (coupling of the palmityl group not only to the hydrazine group of peptide 6, but also to the amino function on the side chain of the lysine residue of said peptide).

Purified lipopeptide 11 is analysed using ES-MS (Micromass Quattro II Electrospray Mass Spectrometer). [M+H]⁺ calculated: 1672.1, found: 1671.6.

30 3) Synthesis of lipopeptides 12, 13, 14, 16 and 18

Procedure is similar to that described in 2) for synthesis of lipopeptide 11, reacting the hydrazinopeptide 6 with compounds 8, 9, 10, 15 and 17, respectively.

35 Only purification of lipopeptide 16 changes. Its purification using HPLC is carried out at a pH of 7.0 in a Zorbax C3 column using the following eluent: from 100% of solvent A (phosphate buffer 50 mM, pH 7.0) to 100% of solvent B

(phosphate buffer 50 mM, pH 7.0, including 50% of isopropanol) in 100 minutes, at the rate of 3 ml/minute and at 50°C, with detection being carried out at 215 nm. Compound 16 thus obtained is then desalted using the following conditions : polystyrene-divinylbenzene column, gradient from 100% of solvent A (water including 0.05% of triethylamine) to 100% of solvent B (20/80 water/ acetonitrile mixture including 0.05% of triethylamine) in 10 minutes, at the rate of 4 ml/minute and at 50°C, with detection taking place at 215 nm.

Characterization of 12, 13, 14, 16 and 18 using ES-MS and the yields obtained for the different lipopeptides are as follows (Table I):

10

Tableau I

lipopeptide	lipophilic group	[M+H] ⁺ calculated	[M+H] ⁺ found	yield
<u>12</u>	oleyl	1697.2	1697.8	53%
<u>13</u>	stearyl	1699.2	1699.5	65%
<u>14</u>	cholesteryl	1845.6	1845.7	56%
<u>16</u>	cis-9,10-epoxystearyl	1713.2	1713.5	53%
<u>18</u>	linoleyl	1695.2	1695.5	51%

Only 6, 7 and 8%, respectively, of diacylated lipopeptides are obtained when synthesizing lipopeptides 12, 13 and 14.

15 **EXAMPLE 4 : Synthesis of lipopeptide 13 by catalytic hydrogenation of lipopeptide 12 (Fig. 4).**

500 µg of palladium on 10% charcoal / coal in suspension in 600 µl of a solution at 20% of acetic acid concentrated in the water are added to 5 mg (2.3 µmoles) of compound 12, obtained in the way described in the previous example, 20 dissolved in 300 µl of the same solution. After 4 h stirring at ambient temperature in a hydrogen atmosphere, 1.64 mg of palladium on coal in suspension in 100 µl of ice cooled acetic acid are added to the reaction mixture. After 20 hours, conversion is total and the medium is filtered on celite and washed with a 20% acetic acid solution in water (3 x 3 ml), and then methanol (3 x 3 ml). The filtrate is concentrated at reduced pressure, deep frozen and then freeze dried. The compound thus obtained is purified using HPLC in a Zorbax C3 column using a linear gradient of 0% to 55% of a water / acetonitrile / TFA mixture (1/4 water / acetonitrile, with 0.05% TFA) in a 0.05% TFA / water mixture (water including 0.05% TFA). The purified compound (2.55 mg, 1.2 mmoles, yield: 52%) is freeze dried and stored at -20°C.

The purity of the purified compound is checked using analytical HPLC in a Zorbax C3 column using the same eluent system as before. The compound is identified by ES-MS : $[M+H]^+$ calculated: 1699.2, found: 1699.6.

EXAMPLE 5 : Synthesis of lipopeptide 21 (Fig. 5).

5

1) Synthesis of hydrazinopeptide 19.

Hydrazinopeptide 19 was synthesized on 0,25 mmol (357.1 mg) of Rink Amide aminomethyl-polystyrene resin including 1% of divinylbenzene (0,70 mmol/g, 100-200 Mesh, Senn Chemicals AG) using the Fmoc/tert-butyl strategy as described, for example, by FIELDS *et al.* *Int. J. Pept. Protein*, 1990, 35, 161, and 10 HBTU/HOBt activation (SCHNÖLZER *et al.* *Int. J. Pept. Protein Res.*, 1992, 40, 180), using an Applied Biosystem 431A peptide synthesizer (Foster City, USA). The Fmoc protective groups are removed using a solution of piperidine at 20% in DMF.

15 The α -NH₂ function is modified using the solid phase electrophilic N-amination procedure developed by C. KLINGER *et al.* (*Tetrahedron Letters*, 1996, 37, 40, 7259-7262). The hydrazinopeptide obtained is deprotected and cleaved from the resin using 10 ml of a TFA solution (94% TFA, 2.5% H₂O, 2.5% thioanisole, 1% triisopropylsilane) for 1h30 with stirring. The compound is then precipitated in 100 ml of an Et₂O/pentane solution (1/1). After precipitation and removal of the supernatant, the pellet is dissolved in 10% acetic acid, deep frozen and freeze dried.

20

The identity of hydrazinopeptide 19 is checked using PDMS-TOF on a Bio-ion 20 plasma desorption mass spectrometer $[M+H]^+$ calculated: 895.5, observed : 895.9.

25 The hydrazinopeptide 19 is purified in a Zorbax C3 preparative column (30°C, detection at 235 nm, buffer A = H₂O 100%/TFA 0.05%, buffer B = isopropyl alcohol 40%/H₂O 60%/TFA 0.05%, flow rate 2 ml/minute, from 0 to 70% in B in 70 minutes). After deep freezing and freeze drying, the hydrazinopeptide 19 is obtained with a yield of 56%. The purity of the product after freeze drying is checked using RP-HPLC under the same conditions as indicated earlier.

30

2) Synthesis of lipopeptide 21.

5.06 mg of hydrazinopeptide 19 are dissolved in 791 μ l of citrate-phosphate buffer, pH 5.11. 1.1 eq. (4,12 μ mol) of succinimidyl palmitate 7 (with Su representing a succinimidyl group) dissolved in 791 μ l of ⁴BuOH are then added. The reaction is monitored using RP-HPLC in a Zorbax C3 column. After 48 h, the reaction mixture is purified in a Zorbax C3 preparative column (30°C, detection at 215 nm, buffer A = H₂O 100%/TFA 0.05%, buffer B = acetonitrile 80%/H₂O 20%/TFA 0.05%, flow rate 3 ml/minute, from 0 to 70% in B in 70 minutes). The lipopeptide 21

yield is 60%.

EXAMPLE 6 : Synthesis of lipopeptide 23 (Fig. 6).

1) Synthesis of hydrazinopeptide 22.

Peptide 22 is prepared on a Fmoc-PAL-PEG-PS resin (0.16 mmol/g, 5 Perseptive) according to the Fmoc/*tert*-butyl strategy and an HBTU/HOBt activation (see example 2) on a Pioneer-Perseptive peptide synthesizer. Protection for the side chains of the amino acids is as follows: His(Trt), Asn (Trt), Glu(O'Bu), Arg(Pbf), Lys(Boc), Ser(Bu). Upon completion of synthesis, the Fmoc group of the α -NH₂ function of the alanine is removed in the presence of piperidine at 20% in the DMF. 10 The N,N'-tri(Boc)hydrazinoacetic acid (1.2 eq.) is then introduced manually using BOP activation *in situ* (BOP: 1.2 eq., DIEA : 3.6 eq. in the DMF for 20 minutes). The peptidyl-resin is washed successively with DMF, dichloromethane, and then ether. It is then dried at reduced pressure for 30 minutes. Cleavage of the peptide-resin link as 15 well as deprotection of the side chains are carried out in the presence of a TFA/phenol/ethanedithiol/thioanisole/H₂O mixture (1 g of dry resin / 10 ml of TFA / 0,25 ml of ethanedithiol / 0.25 ml of H₂O / 025 ml of thioanisole / 0.75 g of phenol) with stirring for 3h30 at ambient temperature. The peptide is precipitated in 200 ml of an Et₂O/heptane mixture (1/1) previously cooled down to 0°C. The precipitate is 20 centrifuged and then dissolved in an H₂O/AcOH mixture (5/1), deep frozen and freeze dried. 263 mg of raw peptide are obtained from 0.072 mmole of resin.

The hydrazinopeptide 22 was purified using HPLC in a Zorbax C3 column using a linear gradient of from 0% to 50% in 70 minutes of a 0.05% TFA/water/isopropanol mixture (2/3) in a 0.05% TFA/water mixture. The purified compound (43 mg) is freeze dried and stored at -20°C. The analysis for

25 hydrazinopeptide 22 using ES-MS is as follows: [M+H]⁺ calculated: 4645.5, found: 4645.7. Its analysis is amino acids is as follows: Lys(3): 2.8; Arg(6): 6.0; Glu(7): 7.3 ; Ala(2): 1.9.

2) Synthesis of lipopeptide 23.

Lipopeptide 23 is obtained from compound 7 and hydrazinopeptide 30 22, according to the mode of operation described earlier in connection with the synthesis of lipopeptide 11. It is obtained with a yield of 40%, after purification. Analysis using ES-MS (Micromass Quattro II Electrospray Mass Spectrometer) of lipopeptide 23 gives the following results: [M+H]⁺ calculated: 4883.5, found: 4883.7.

EXAMPLE 7 : Analysis and quantification of the expression of the molecules of

35 **class II of the Major Histocompatibility Complex (MHC), at the surface of the** celis, induced by incubation of these celis with modified peptides according to the

invention.

1) Peptides and lipopeptides used.

Use is made of the peptides named Mu-Gly, MuSc-Gly, 22 and 22Sc, as well as lipopeptides Mu-Gly-palm, MuSc-Gly-palm, 23 and 23Sc. The term 5 « Sc » designates a "scramble" version of the peptide; that is to say a peptide sequence in which the amino acids (according to their order in the sequence) have been mixed. The term "palm" designates a palmitoyl group.

Peptide 22 and lipopeptide 23 were prepared as indicated in example 6. Peptide 22Sc ("scramble" version of peptide 22) has the following sequence:

10 H₂N-NH-CH₂CO-PSRENQNAVKIQQLSVVLRREQKHRVERLAFRNQLPF-NH₂

Peptide 22Sc was prepared on a Rink amide MBHA PS resin (0.25 mmole, 0.74 mmol/g, Applied Biosystems, Foster City, USA) according to the Fmoc/tert-butyl strategy, on an Applied Biosystem 430 A peptide synthesizer. Upon completion of synthesis and after deprotection of the N-terminal amino group, half of 15 the peptidyl-resin (125 μ moles) is treated in the presence of 63.4 mg (162 μ moles) of N,N'-tri(Boc)hydrazinoacetic acid, 84.6 mg (162 μ moles) of PyBOP and 85.2 μ l (486 μ moles) of DIEA in the DMF for 30 minutes. Cleavage and deprotection of peptide 22Sc was carried out in the presence of a TFA/H₂O/phenol/EDT/thioanisole mixture (1g of dry resin /10.0 ml of TFA/0.5 ml of H₂O/0.75g of phenol /0.25 ml of EDT/0.5 20 ml de thioanisole). After precipitation in an ether/heptane mixture (1/1), centrifuging, deep freezing and freeze drying, 22Sc was purified using RP-HPLC as peptide 22. Once freeze dried, the peptide 22Sc (117.5 mg, 15.9%) was stored at -20°C. Its analysis in amino acids is as follows: Lys(3): 2.6; Arg(6): 6.0; Glu(7): 7.4; Ala(2): 1.9. Its analysis using ES-MS is as follows: [M+H]⁺ calculated 4645.4; found 4645.6.

25 Peptide 23Sc (the "scramble" version of peptide 23) has the following sequence: palm-NH-NH-CH₂CO-PSRENQNAVKIQQLSVVLRREQKHR-VERLAFRNQLPF-NH₂. Peptide 23Sc was prepared in the same way as peptide 23, but from peptide 22Sc instead of from peptide 22. Coupling was carried out using 15.27 mg of peptide 22Sc. The peptide 23Sc was purified in a Zorbax C3 preparative 30 column using a linear gradient of from 20 to 60%, in 100 minutes, of a solvent B (acetonitrile 80%/water 20%/TFA 0.05%) in a solvent A (water/TFA 0.05%). The temperature is 50°C, the elution rate is 3 ml/minute and detection is carried out at 215 nm. After deep freezing and freeze drying, 2.46 mg (yield of 16%) of peptide 23Sc were thus obtained. The purity of this purified product is checked using RP- 35 HPLC in a Zorbax C3 column using a gradient of 0 to 100%, in 60 minutes, of solvent B in solvent A (1 ml/minute, 215 nm, 50°C). A cross check is carried out by capillary

electrophoresis using a citrate buffer, 20 mM, pH 3.0 at 40°C, 30 kV over 10 minutes. Analysis of product 23Sc using ES-MS is as follows: [M+H]⁺ calculated 4883.8; found 4882.5.

Peptides and lipopeptides Mu-Gly, MuSc-Gly, Mu-Gly-palm and 5 MuSc-Gly-palm have the following sequences respectively:

Mu-Gly : H₂NCH₂CO-AKFEVNNPQVQRQAFNELIR-
VVHQLLPESSLRKRKRSR-NH₂

MuSc-Gly : H₂N-CH₂CO-PSRENQNAVQIQKLSVVLR-
REQKHRVERLAFRNQSLPF-NH₂

10 Mu-Gly-palm : palm-NHCH₂CO-AKFEVNNPQVQRQAFNELIR-
VVHQLLPESSLRKRKRSR-NH₂

MuSc-Gly-palm : palm-NHCH₂CO-PSRENQNAVQIQKLSVVLR-
REQKHRVERLAFRNQSLPF-NH₂

These peptides were obtained by solid phase peptide synthesis 15 according to the standard protocols, using the Fmoc/*tert*-butyl strategy. In the case of lipopeptides Mu-Gly-palm and MuSc-Gly-palm, after elongation of the peptide sequence and deprotection of the glycine in N-terminal position with the piperidine at 20% in the DMF, the palmitic acid (4 eq.) was linked using an HBTU/HOBt/DIEA activation: 4 eq / 4 eq / 12 eq (equivalents in relation to the amino functions) in the 20 DMF for 40 minutes. The peptides were cleaved and deprotected in the presence of a TFA/H₂O/phenol/EDT/thioanisole mixture (1g of dry resin /10.0 ml of TFA /0.5 ml of H₂O/0.75g of phenol/0.25 ml of EDT/0.5 ml of thioanisole). The peptides and lipopeptides were purified prior to use.

Thus, peptides 22, 23, Mu-Gly and Mu-Gly-palm include the same 25 peptide sequence of 38 amino acids. They differ in that they include, or do not include, a fatty chain of palmitic acid and with regard to the nature of the chemical bond of the fatty chain to the peptide sequence.

2) Protocol for quantification of the expression of the molecules of Class II of the Major Histocompatibility Complex (MHC) at the surface of the cells.

30 The COLO 205 cells (human cell line from a carcinoma of the colon) are from the ATCC (American Type Culture Collection) cell bank. They are cultivated in an RPMI 1640 medium (Gibco BRL, Courbevoie, France) with 10% of fetal calf serum (SVF#) and 5 mM of sodium pyruvate, and incubated at 37°C in the presence of 5% of CO₂. The cells are stimulated for 24 hours with different 35 concentrations (35, 50 or 65 μ M) of peptides or lipopeptides. The cells are then marked for 1 hour at 4°C with 10 μ l of anti-HLA DR class II mouse antibody, linked

to the FITC ((TAL clone, 1B5, Cymbus Biotechnology Ltd, Hants, Grande-Bretagne) in 10% of SVF/PBS, and then washed 3 times with SVF/PBS.

The surface expression of the class II molecules of the MHC is analyzed by flow cytometry using a Coulter EPICS II cytometer, at the rate of 10 000 events per sample. The fluorescence intensity observed is directly proportional to the quantity of molecules of class II of the MHC present at the surface of the cells. The fluorescence mean observed for the non-treated cells and for the treated cells ("Mean" column in Table II) makes it possible to calculate a ratio between the fluorescence mean for the treated cells and the fluorescence mean for the non-treated cells ("Ratio" column in Table II).

3) Results.

The results obtained are collected in Table II.

Table II

15

Name of product	35 μ M		50 μ M		65 μ M	
	Mean	Ratio	Mean	Ratio	Mean	Ratio
Mu-Gly-palm	6.53	8.9	6.43	8.45	2.71	3.6
MuSc-Gly-palm	7.23	9.86	7.45	10.16	9.76	13.1
Mu-Gly	1.40	1.9	1.31	1.78	1.93	2.6
MuSc-Gly	1.47	2	1.69	2.3	1.82	2.48
<u>23</u>	7.35	10.2	8.02	10.94	13.3	18.14
<u>23Sc</u>	4.72	9.16	4.51	6.15	5.55	7.5
<u>22</u>	1.13	1.5	1.11	1.5	1.25	1.70
<u>22Sc</u>	1.36	1.8	1.60	2.18	1.67	2.27

It will be noted that the products not derivatized by a palmitic chain (peptides Mu-Gly and 22) do not lead to an increase in the expression of MHC II, by comparison with the « scramble » versions, namely MuSc-Gly and 22Sc, respectively.

20 On the other hand, lipopeptide 23 induces a dose-dependent expression of MHC II by comparison with the "scramble" version 23Sc. This expression is thus specific to the sequence and the presence of the lipophilic chain linked to the peptide via a hydrazide link. Thus the coupling in solution of a hydrazinopeptide with an activated fatty acid

to obtain the corresponding lipopeptide leads to a product that is capable of passing through the membranes of the cells and of reaching an intracytoplasmic target.

Lipopeptide 23 gives results that are better than those of the lipopeptide synthesized in the standard way (Mu-Gly-palm). Other experiments have
 5 shown that the results were comparable between lipopeptides 23 and Mu-Gly-palm. However, the purity of lipopeptide 23 is far greater than that of lipopeptide Mu-Gly-palm. In addition, inspection of the cells treated with the lipopeptides shows that lipopeptide 23 according to the invention is far less cytotoxic for the cells than standard peptide Mu-Gly-palm.

10 **EXAMPLE 8: Use of N,N'-di(Boc)hydrazinoacetic acid for functionalizing a peptide with an α -hydrazinoacetic group**

The peptidyl-resin of formula Fmoc-G-R(Pmc)-K(Boc)-R(Pmc)-S(tBu)-H(Trt)-A-G-Y(tBu)-Q(Trt)-T(tBu)-I-O-resin was solid phase synthesized according to the Fmoc/*tert*-butyl strategy using a Wang resin, as described in example
 15 2. 143.9 mg (33.7 μ mol) of this peptidyl-resin are treated, for 2 minutes and then for 20 minutes, with the piperidine/NMP mixture (20/80) in order to deprotect the terminal amino function.

11.7 mg N,N'-di(Boc)hydrazinoacetic acid (40.4 μ mol, 1.2 eq.) are solubilized in 500 μ l of NMP in the presence of 5.5 mg of HOBt (40.4 μ mol, 1.2 eq.)
 20 and of 15.3 mg of HBTU (40.4 μ mol, 1.2 eq.). The addition of 21 μ l of DIEA leads to the production of hydroxybenzotriazole ester that is activated and non-isolated and immediately added to the peptidyl-resin. After 45 minutes' coupling, the Kaiser test (*Anal. Biochem.*, 1970, 34, 595) is still positive. A second coupling is thus carried out, under the same conditions, and this time, a negative Kaiser test is obtained.

25 The peptidyl-resin is treated with 1.5 ml of a TFA/phenol/EDT/thioanisole/water mixture (10 ml/0.75 g/0.25 ml/0.25 ml/0.5 ml) during 1 h 30. The peptide is precipitated in an ethyl ether / heptane mixture cooled down to -20°C (2 x 20 ml). The supernatant is removed. The precipitated peptide is re-solubilized in 1 ml of acetic acid. 5 ml of water are then added, the solution is degassed with nitrogen, deep frozen and freeze dried. The raw peptide obtained is purified using RP-HPLC in a Nucléosil C18 column. Eluent A: TFA 0.05% in water. Eluent B: water/propan-2-ol (60/40) including 0.05% of TFA. The gradient is as follows: from 0 to 60% of B in 60 minutes, the product of interest being eluted in 23 minutes. After purification, 19.9 mg of pure peptide are obtained (yield: 29.7%), functionalized at its
 30 N-terminal end by an α -hydrazinoacetic function. Its analysis using ES-MS is as follows: [M+H]⁺ calculated: 1545.6; found: 1545.4.